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DIFFERENCES IN STARFISH OOCYTE SUSCEPTIBILITY TO POLYSPERMY DURING THE COURSE OF MATURATION[1]

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It is known that the eggs of marine invertebrates such as the sea urchin and starfish lose the capability to fertilize or to develop normally when they are maintained in sea water for too long a period after being shed (Goldforb, 1935; Clark, 1936). In starfish oocytes, the optimum period for insemination is between germinal vesicle breakdown and the formation of the first polar body (Fol, 1879; Delage, 1901); insemination after the formation of the first polar body results in a decreased rate of subsequent normal development (Lillie, 1915).

Some earlier investigators reported that starfish oocytes, when inseminated in sea water for several hours, lose their capacity to resist polyspermy (Chambers, 1923; Clark, 1936). Thus, the decreased rate of normal development after the formation of the first polar body may be related to polyspermy. On the other hand, Chambers and Chambers (1949) have suggested that during the course of oocyte maturation, there is a specific period of ripening of cytoplasm when insemination would result in normal fertilization and development; after this period there is a decline in proper functional interrelation between the sperm and cytoplasm.

The present paper reports findings in support of the work of Chambers (1923) and Clark (1936).

Materials and Methods

Oocytes from *Asterina pectinifera* and *Asterias amurensis* were used. The starfish were collected at the seashore near the Marine Biological Station of Tohoku University, Asamushi. Specimens of *Asterina pectinifera* were collected in September and kept in an aquarium supplied with circulating cold sea water at 10 to 13° C for two months. Specimens of *Asterias amurensis* were collected in April and maintained in running sea water tanks at 7 to 9° C for a month in the laboratory.

Asterina pectinifera. All but a few of these oocytes showed no conspicuous change when isolated from the ovary in sea water. The isolated full-grown oocytes with germinal vesicles were prepared by tearing the ovaries with forceps in sea water and washed three times with sea water. The oocytes were kept for at least 1 hr to make sure that they did not undergo spontaneous maturation.

Asterias amurensis. These oocytes usually undergo spontaneous maturation when

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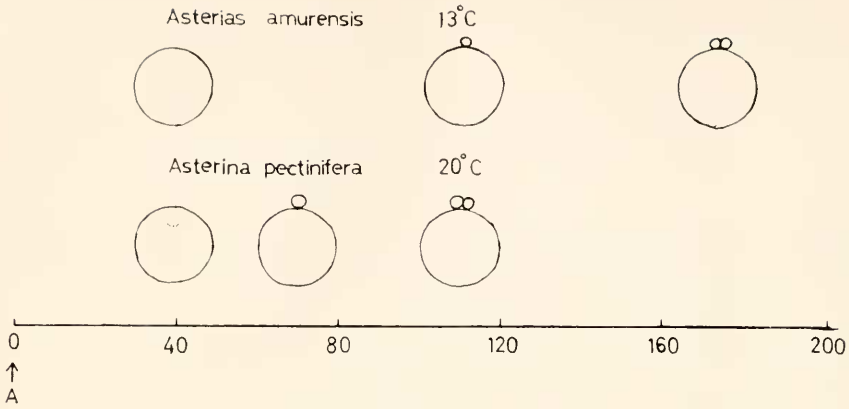

FIGURE 1. The time course of oocyte maturation in *Asterina pectinifera* and *Asterias amurensis*. This shows the time (min) when germinal vesicle breakdown occurs, and the first and second polar body form. 1-Methyladenine was added at A.

isolated in sea water. To obtain oocytes, ovaries were directly immersed in 1-methyladenine sea water. Full-grown oocytes which began germinal vesicle breakdown were spawned and these oocytes were used.

1-Methyladenine. 1-Methyladenine (Sigma) dissolved in deionized water at a concentration of 10^{-3} M was served as stock solution, and diluted with sea water to a concentration of 10^{-6} M before use.

Schedule of oocyte maturation. As shown in Figure 1, in an oocyte of *Asterina pectinifera,* the germinal vesicle disappears completely 40 min after being placed in 1-methyladenine sea water (10^{-6} M) and the first polar body forms at 65 to 75 min and the second polar body at 105 to 115 min, respectively at 20° C. In *Asterias amurensis,* oocytes which began germinal vesicle breakdown were released from ovary 40 min after ovary was immersed in 1-methyladenine sea water (10^{-6} M). The first polar body forms at 105 to 115 min and the second polar body at 170 to 180 min at 12° C.

Spermatozoa. To obtain a sperm suspension, an isolated testis was torn with forceps in an empty Petri dish. Before use, 10 μl of semen was sucked up into a micropipette and diluted into 10 ml of 10^{-4} M histidine sea water to increase the sperms' motility. These sperm suspensions were diluted from 10-fold to 100,000-fold with fresh sea water and the number of spermatozoa was counted with haematometer. Inseminations were carried out by mixing 9 ml of oocyte suspension with 1 ml of sperm suspension.

Cytological procedures. Examined oocytes were fixed with Carnoy's solution and were sectioned by the usual paraffin method. The sections, 10 μ in thickness, were stained with Feulgen's reaction and counterstained with fast green.

Experimental procedures. The capacity of oocytes for normal development was investigated as follows; oocytes of *Asterina pectinifera* were used. Isolated immature oocytes were immersed in 1-methyladenine sea water (10^{-6} M) at 20° C and then, one part of these oocytes was transferred to another Petri dish containing fresh sea water and inseminated at one period, the other part was transferred to another Petri dish and inseminated at another period, respectivly, so on. Oocytes were 2000 to 2500 per 10 ml sea water and concentration of sperm was finally 2×10^6 per ml. The number of the oocytes which formed fertilization membranes was counted 5 min after each insemination and then these oocytes were kept for 20 hr at 20° C. The oocytes were allowed to develop to early gastrula and the number was counted under a light microscope.

The occurrence of normal first cleavage was checked as follows: the ovaries of specimens of *Asterias amurensis* were directly immersed in 1-methyladenine sea water (10^{-6} M) and one part of released oocytes (2000–2500) was transferred to Petri dishes containing 10 ml fresh sea water at 40, 110, 150, and 180 min after the immersion in 1-methyladenine sea water and inseminated respectively. The concentration of sperm used was finally 8×10^5 per ml. Formation of fertilization membrane and first cleavage were observed 2.5 hr after each insemination. This experiment was performed at 12° C.

The relation between occurrence of abnormal cleavage and sperm concentration was examined as follows: oocytes of *Asterias amurensis* (2000–2500) were transferred to Petri dishes containing 10 ml fresh sea water at 40 and 180 min after addition of 1-methyladenine sea water (10^{-6} M) and inseminated with various sperm concentrations which were from 8×10^1 to 8×10^6 per ml, respectively. Formation of fertilization membrane and first cleavage were checked at 2.5 hr after each insemination. This experiment was done at 12° C.

Results

Oocytes of *Asterina pectinifera* were immersed in 1-methyladenine sea water for various periods ranging from 40 to 180 min. The oocytes were inseminated in fresh sea water, at various intervals, after being placed in 1-methyladenine sea water. The formation of fertilization membrane was checked 5 min after insemination, and then the oocytes were continuously kept in sea water for 20 hr; at the end of the experiment the number of oocytes which developed to early gastrula was counted under a light microscope.

When inseminations were performed between 40 and 80 min after being placed in 1-methyladenine sea water, 73% of the oocytes developed to early gastrula. Forty-five per cent developed to gastrula, when inseminated at 120 min (period of the formation of second polar body) and only 2% at 180 min, respectively (Fig. 2).

Usually, germinal vesicle breakdown occurs spontaneously in a few per cent of the oocytes after isolation in sea water without 1-methyladenine application in *Asterina pectinifera* oocytes. In order to synchronize the course of oocyte maturation, isolated oocytes were kept in sea water for 1 hr and then spontaneously matured oocytes were checked. In this experiment, the mean rate of spontaneous maturation was 19%. For these spontaneously matured oocytes, about 100 min had

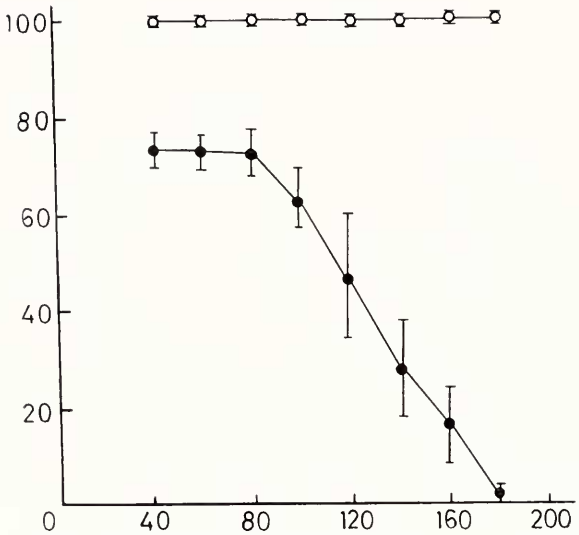

FIGURE 2. The percentage of fertilization membrane formation and normal early gastrula in *Asterina pectinifera* oocytes. Abscissa: time (min) after 1-methyladenine application; ordinate: the rate (%) of fertilization membrane and normal early gastrula formation. Open circles, fertilization membrane formation; closed circles, normal early gastrula. Each point shows mean ± of five experiments.

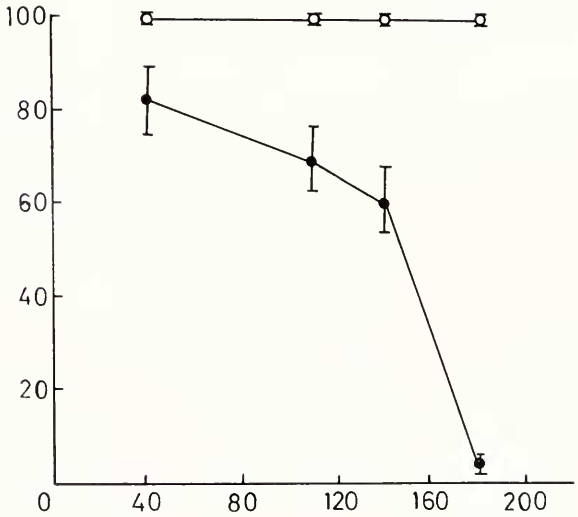

FIGURE 3. The percentage of fertilization membrane formation and normal first cleavage in *Asterias amurensis* oocytes. Abscissa: time (min) after 1-methyladenine application; ordinate: the rate (%) of fertilization membrane formation and normal first cleavage. Open circles, fertilization membrane formation; closed circles, normal first cleavage. Each point shows mean ± of four experiments.

already elapsed after isolation in sea water, before insemination, and these spontaneously matured oocytes developed abnormally upon insemination. Subsequently, the maximum rate of oocytes which developed to early gastrula did not exceed 73% even in case of the insemination at 40 min.

Next, the occurrence of first cleavage was checked 2.5 hr after insemination. In this experiment, oocytes of *Asterias amurensis* were used. After being placed in 1-methyladenine sea water for various times, oocytes were transferred to fresh sea water and inseminated. Although the formation of fertilization membrane occurred in all oocytes, the rate of normal first cleavage decreased with increased intervals after being placed in 1-methyladenine sea water; 82%, when inseminated at 40 min, 69% at 110 min (period of first polar body formation) and only 4% at 180 min (period of second polar body formation) (Figs. 3, 4).

In addition, these abnormal cleavages occurred even if 1-methyladenine was completely removed by washing with fresh sea water after occurrence of germinal

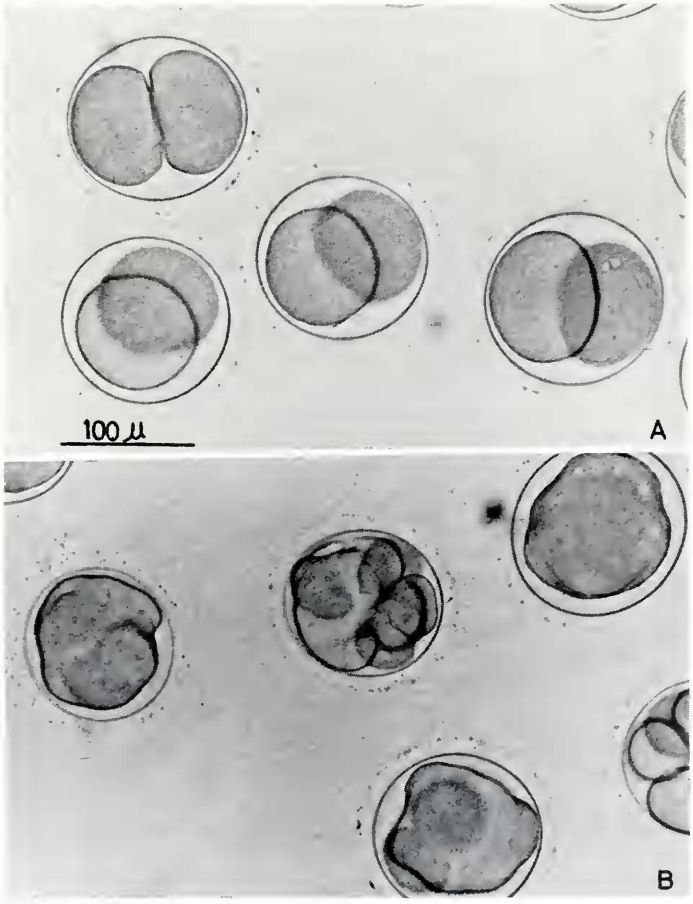

FIGURE 4. Normal first cleavage and abnormal cleavage in *Asterias amurensis* oocytes when inseminated at 40 min (A) and 180 min (B) after 1-methyladenine application.

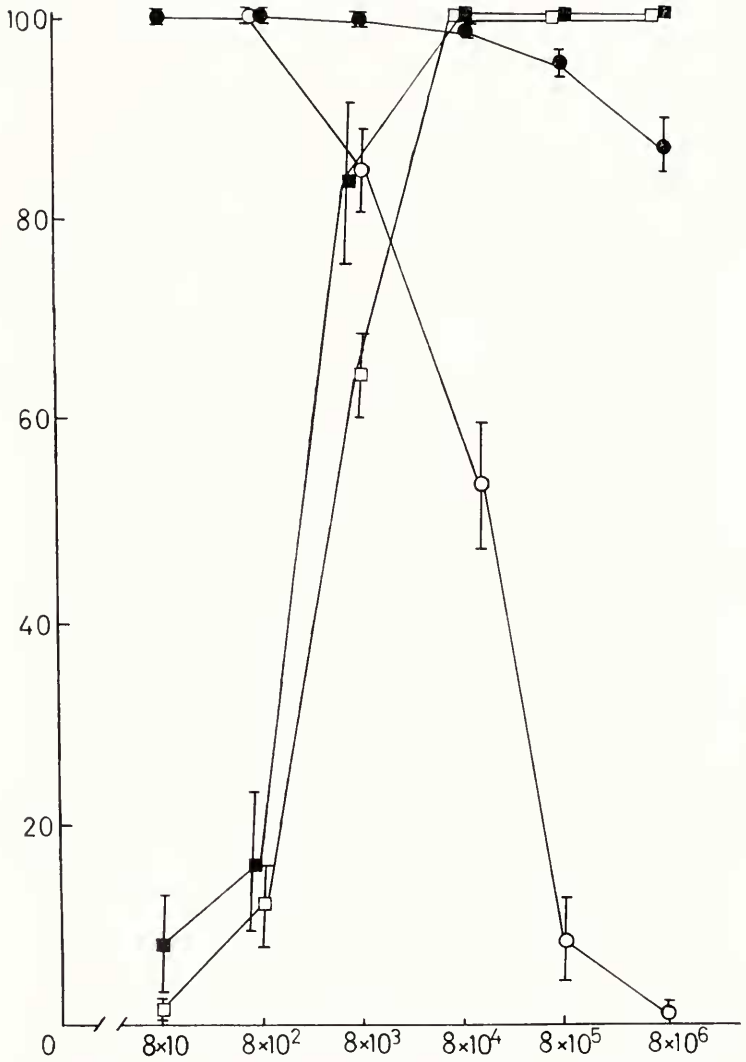

FIGURE 5. Relation between sperm concentration and normal first cleavage. Abscissa: sperm concentration (per ml); ordinate: the rate (%) of fertilization membrane formation and normal first cleavage. Solid squares, fertilization membrane formation upon insemination at 40 min; open squares, fertilization membrane formation upon insemination at 180 min; closed circles, normal first cleavage upon insemination at 40 min; open circles, normal first cleavage upon insemination at 180 min. Each point shows mean ± of five experiments.

vesicle breakdown. Therefore, this abnormality was not due to the excess of 1-methyladenine.

Next, oocytes of *Asterias amurensis* were inseminated at 40 and 180 min after being placed in 1-methyladenine sea water, with various sperm concentrations ranging from 8×10^3 to 8×10^6 per ml. When inseminations were performed

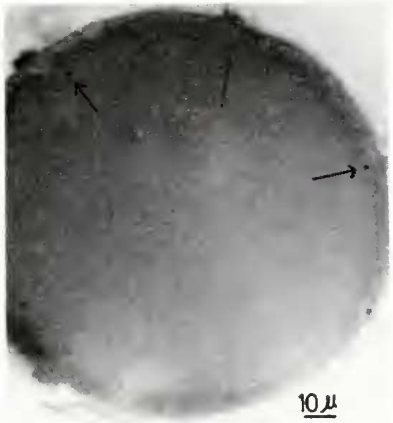

FIGURE 6. Section through an *Asterina pectinifera* oocyte which was inseminated at 180 min after 1-methyladenine application. Arrows show sperm nuclei.

40 min after being placed in 1-methyladenine sea water, the rate of fertilization membrane formation increased with the increased sperm concentrations. The rate of normal first cleavage showed only a slight decrease with higher sperm concentrations (Fig. 5); 87% of these oocytes cleaved normally when inseminated with heavy sperm concentration (8×10^6 per ml). On the other hand, when inseminations were performed at 180 min (period of second polar body formation), the rate of normal first cleavage showed a sharp decrease accompanying the increased sperm concentrations, although the rate of fertilization membrane formation showed a similar curve seen at 40 min: the rate of normal first cleavage was 53%, with moderate sperm concentration (8×10^4 per ml) which brought normal cleavage for almost all oocytes that were inseminated at 40 min, 8% with 8×10^5 per ml and only 1% with heavy sperm concentration (8×10^6 per ml). These results showed that the increase of abnormality in the oocytes inseminated at 180 min was due to the increased sperm concentration. Therefore, it seemed that polyspermy occurred in the oocytes inseminated at 180 min. This probability was confirmed cytologically (Fig. 6). Oocytes of *Asterina pectinifera* were inseminated 120 min after being placed in 1-methyladenine sea water with a moderate sperm concentration (5×10^5 per ml), and 5 min after insemination they were fixed with Carnoy's solution. Microscopic examination showed that most oocytes contained more than two sperm nuclei.

In addition, when insemination was performed with moderate sperm concentration (8×10^4 per ml) at the period of second polar body formation, the rate of fertilization membrane formation did not attain one hundred per cent (Fig. 5), but about 50% of the fertilized oocytes showed normal first cleavage and most of them developed to early normal bipinnaria.

DISCUSSION

During the course of starfish oocyte maturation, the optimum period for insemination is between germinal vesicle breakdown and first polar body formation

(Fol, 1879; Delage, 1901). Insemination after first polar body formation results in a tendency toward a decline of normal development; abnormality becomes more pronounced when oocytes are inseminated 30 to 60 min after second polar body formation (Lillie, 1915). But in these studies, the cause for occurrence of abnormality was not clarified. In our results, when inseminations were performed between germinal vesicle breakdown and first polar body formation, fertilization membrane formed normally in all cases and the first cleavage occurred normally in most oocytes. However, when inseminations were performed after first polar body formation, the rate of first cleavages and subsequent development apparently decreased. Only a small percentage of oocytes underwent normal cleavage when inseminated after second polar body formation, although fertilization membrane was formed normally in all oocytes. Figure 5 shows the increased rate of abnormal cleavage accompanying increased sperm concentrations in oocytes with second polar body: with moderate sperm concentration (8×10^4 per ml) the rate of normal cleavage was 53%, but with high sperm concentration (8×10^6 per ml), the rate of normal cleavage was only one per cent. However, even at the same high sperm concentration, normal cleavage occurred in 87% of the oocytes which completed germinal vesicle breakdown. Thus, these results suggested that the increased rate of abnormal cleavage was due to the occurrence of polyspermy. The occurrence of polyspermy was confirmed by cytological observation.

Chambers and Chambers (1949) have suggested that there is a decline in proper functional interactions between sperm and egg cytoplasm when insemination is performed after the optimum ripening period. In the present study, however, some fertilized oocytes, which were inseminated after second polar body formation, showed normal cleavage and developed to early bipinnaria if the sperm concentration was light. It is suggested that as long as polyspermy was prevented, the oocyte remained viable, even after second polar body formation.

Earlier investigators reported that starfish oocytes become susceptible to polyspermic fertilization after standing in sea water less than two hrs (Chambers, 1923; Clark, 1936). In their studies, the occurrence of polyspermy and abnormal development were not considered in relation to various periods of oocyte maturation. Our study confirms that the number of starfish oocytes showing polyspermy gradually increases with increasing time intervals between first polar body formation and sperm addition, and that almost all the oocytes show polyspermy when the insemination is performed after second polar body formation. Even in the latter case, however, fertilization membrane is normally formed. It is postulated that polyspermy occurs before fertilization membrane formation and that there is some mechanism(s) of protection against polyspermy between the first sperm entry and fertilization membrane formation. The mechanism(s) may be gradually lost after first polar body formation and disappear completely after second polar body formation.

On the other hand, polyspermy occurs regardless of the fertilization membrane formation in immature oocytes which have intact germinal vesicles (Cayer, Kishimoto and Kanatani, 1975; Hirai, 1976). Thus, the protective mechanism(s) against polyspermy may be acquired during the maturation process.

In sea urchin eggs, it was recently demonstrated by Jaffe (1976) that polyspermy was quickly inhibited as a result of electrical depolarization brought about

by the entry of the first spermatozoon. In starfish oocytes, it was reported that potassium conductance of oocyte membrane changed during maturation, thus altering the form of action potential subsequently generated (Miyazaki, Ohmori and Sasaki, 1975). Potassium conductance was small before first polar body formation, and larger after it (Miyazaki, Ohmori and Sasaki, 1975). It is suggested that the change of potassium conductance during oocyte maturation may affect electrical depolarization brought about by the entry of the first spermatozoon. Further examination would be desirable.

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Summary

During the course of starfish oocyte maturation, the optimum period for insemination is between germinal vesicle breakdown and the first polar body formation. The rate of polyspermy increases with increasing time intervals between the first polar body formation and sperm addition, although the fertilization membrane is formed normally in all oocytes. As long as polyspermy is prevented, however, the oocytes remain viable even after the formation of second polar bodies. Thus, it is postulated that there is some mechanism(s) of protection against polyspermy between the first sperm entry and the fertilization membrane formation. The mechanism(s) may be gradually lost after the first polar body formation and disappear completely after the second polar body formation.

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